Using an animal model knocked in for the $RYR1_{Y522S}$ mutation we observed that bleeding times were increased by more than two fold in heterozygous mice compared to their wild type littermates, with no differences in platelet numbers nor aggregation characteristics between heterozygous $RYR1_{Y522S}$ carriers and control littermates. Bleeding abnormalities have also been seen in some patients with dominant *RYR1* mutations; as part of a comprehensive study we investigated in detail RyR1 expression in smooth muscle cells isolated from different tissues and if there is a causal link between *RYR1* mutations and prolonged bleeding times.

2274-Pos Board B293

Modeling a Ryanodine Receptor Amino-Terminal Domain Connecting the Central Vestibule and the Corner Clamp Region

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Ryanodine receptors (RyRs) form a class of intracellular calcium release channels in various excitable tissues and cells such as muscles and neurons. They are the major cellular mediators of the release of calcium ions from the sarcoplasmic reticulum, an essential step in muscle excitation-contraction coupling. Several crystal structures of skeletal muscle RyR1 peptide fragments have been solved, but these cover less than 15% of the full-length RyR1 sequence. In the present study, we obtain pseudo-atomic models for RyR fragments, consisting of residues 850-1,056 in rabbit RyR1 or residues 861-1,067 in mouse RyR2, by combining modeling techniques with sub-nanometer resolution cryo-electron microscopy (cryo-EM) maps. These fragments dock into a domain that connects the central vestibule and corner clamp region of RyR, with a good match between the secondary structure elements in the cryo-EM map and the pseudo-atomic models, and also consistent with our previous results of 3D cryo-EM RyR-GFP mapping and FRET measurement between RyR and FKBP. A combined model of the RyR fragment and FKBP docked into the cryo-EM map suggests that the fragment is positioned adjacent to the FKBP binding site. Its predicted binding interface with FKBP consists of primarily electrostatic contacts and contains several disease-associated mutations. A dynamic interaction between the fragment and a RyR phosphorylation domain, characterized using FRET data, also support the structural predictions of the pseudo-atomic models.

2275-Pos Board B294

Structural Insight into the Phosphorylation Domain in Ryanodine Receptors

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Ryanodine receptors (RyRs) are large Ca^{2+}-release channels located in the SR membrane. They play a central role in the excitation-contraction coupling of skeletal (RyR1) and cardiac (RyR2) muscle. Over 500 disease mutations have been identified in RyRs that can cause several skeletal muscle disorders and cardiac arrhythmias. RyRs are also the target for phosphorylation, most notably by CaMKII and PKA. Although a lot of controversy surrounds these events, several studies indicate that phosphorylation can upregulate RyR activity. Here we present crystal structures of a domain in all three different RyR isoforms, containing the Ser2843 (RyR1) and Ser2808/Ser2814 (RyR2) phosphorylation sites. There are 11 disease mutations located in the RyR1 domain, several of which cluster near the phosphorylation site, suggesting that phosphorylation and disease mutations may affect the same interface. Crystal structures of the disease mutants show that the mutations affect either surface properties or intradomain salt bridges. One mutation, L2867G, causes a drastic reduction of thermal stability of the domain, and results in aggregation at room temperature. In vitro phosphorylation experiments identify several novel PKA and CaMKII phosphorylation sites in the same loop region of the RyR2 phosphorylation domain, also supported by another recent in vivo study. The observation of simultaneous phosphorylation events on multiple sites indicates that RyRs may be truly 'hyperphosphorylated'. Docking into cryo-electron microscopy maps locates the domain in the clamp region, a region that has been shown to couple allosterically to channel opening. Disease mutations and phosphorylation may therefore cause conformational changes that affect the allosteric coupling and facilitate channel opening.

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Analysis of Fluorescence Microscopy Super-Resolution Data of Protein Assemblies

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In super-resolution microscopy applications, previously unknown fluorescent signal patterns are recorded. The interpretation of such nanoscale data is often unexpectedly complex, and can be performed by different analysis strategies: 1) empirical statistics of the spatial distribution of intensity values to identify local objects, 2) inverse problem approaches to convert signals into objects based on external data models or other external assumptions, 3) decomposition of spatial signal patterns into spatial modes.

Here, we compare and relate two distinct approaches of analysing STED microscopy images of RyR2 clusters (cardiac ryanodine receptor type 2). RyR2 Ca^{2+} release channels are essential for heart muscle function (excitation-contraction coupling). Yet, the nature of lateral channel organization within super-structural clusters is unknown and important for models of local control mechanisms of RyR2 Ca^{2+} release activity.

We established multi-scale analysis of RyR2 signal patterns employing wavelet analysis. This analysis decomposes the initial image with predefined wavelets into spatial modes identifying dominant scales of signal fluctuations. We tested the sensitivity of this approach for different wavelets with artificial and modified images. Distinct scales represent inter-cluster spacing and intra-cluster patterns, respectively. Furthermore, we compare the spatial mode analysis with object-based approaches. For object-based analysis, RyR2 cluster substructures were identified with a multi-step thresholding procedure. After increasing the threshold level step-by-step, the hierarchy of the segmentation output was analysed with logical operators. Accordingly, we identified cluster sub-structures as discrete objects of variable sizes with typical spacings ranging from 78 to 128 nm (IQR range) that we interpret as individual cluster building-blocks.

We conclude that identification of common protein cluster building principles in highly variable signal structures as typical for RyR2 clusters benefits from combining object-based approaches with spatial mode analysis.

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Gating of the Pore of the Human Ryanodine Receptor Type 2 does not require Glycine Hinges

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Many potassium channels use a conserved glycine residue to allow flexibility of the inner helices and facilitate gating. A KcsA-based analogy model of the RyR2 pore-forming region (Welch et al Biophys J 87: 2335-2351) indicates that this region is composed of structural elements equivalent to those found in potassium channels and includes a potentially equivalent inner-helix hinge motif (GXXXXA).

We studied the functional consequences of substitutions of the conserved glycine residue at position 4864 in recombinantly expressed human RyR2 GFP-tagged proteins. Whilst wild type (WT) RyR2 and the mutants G4864A and G4864V were expressed at equivalent levels in HEK293 cells, G4864P was less tolerated with a reduced expression. This was confirmed by western blot analysis in HEK293 membrane preparations.

Caffeine-induced intracellular calcium release was observed in Fluo3-loaded HEK293 cells expressing WT, G4864A and G4864P RyR2 channels. In contrast cells expressing G4864V were not sensitive to caffeine. [3H]-ryanodine binding to HEK293 membrane preparations resulted in similar specific binding for WT and G4864A at 0.192 $\pm$ 0.005 and 0.190 $\pm$ 0.002 pmol/mg, while G4864V and G4864P showed comparable low values of 0.006 $\pm$ 0.001 and 0.002 $\pm$ 0.002 pmol/mg, respectively. Characteristic single channel current fluctuations were observed following the incorporation of purified WT and G4864A RyR2 proteins into planar phospholipid bilayers and G4864A channels displayed ion handling, and calcium-dependent gating properties comparable to WT channels. No equivalent activity was observed for G4864V and G4864P.

Our investigation indicates that whilst functionally sensitive to mutagenesis, a glycine residue at 4864 is not essential for RyR2 channel gating. Rather, there is a requirement for amino acids with small side chains that allow close packing of helices during transitions from closed to open states. The BHF supported this research.

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Structure of Glutaraldehyde Cross-Linked Ryanodine Receptor

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The ryanodine receptor (RyR) and dihydropyridine receptor (DHPR) along with several other smaller proteins form a large and dynamic complex, often referred to as the excitation-contraction coupling machinery. Complexes of

RyR1 and its protein ligands are often not stable in vitro and therefore difficult to study by single-particle cryo-electron microscopy (cryo-EM). The main goal of this study was to develop a procedure to cross-link protein ligands to RyR1 and visualize these complexes by single-particle cryo-EM. To test the cross-linking protocol we used the complex of FK506-binding protein and RyR1 (FKBP12:RyR1) as a model system, since the structure of FKBP12:RyR1 is known. Glutaraldehyde quantitatively cross-linked RyR1 subunits to each other and FKBP12 to RyR1 without damaging the ultrastructure. Cross-linked FKBP12:RyR1 was visualized in 2D averages, and was identical to that of previously published non-cross-linked FKBP12:RyR1. The effect of glutaraldehyde cross-linking on RyR1 structure was characterized using 3D single-particle cryo-EM and by [3H]ryanodine binding assay. Glutaraldehyde cross-linking preserved the gross morphology of RyR1, but induced minor structural changes at the cytoplasmic and transmembrane regions of RyR1. Glutaraldehyde cross-linking enhanced [3H]ryanodine binding to RyR1 by ~30%. Based on these results we propose that cross-linking RyR1 subunits by glutaraldehyde locked RyR1 in an open-like conformation state.

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FRET-based Structural Measurements of the Type 1 Ryanodine Receptor using Site-Specific Fluorophore Labeling to Tetracysteine Motifs

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The type 1 ryanodine receptor (RyR1) is an intracellular Ca^{2+} release channel that mediates skeletal muscle excitation contraction coupling. While the overall shape of RyR1 has been elucidated using cryo electron microscopic reconstructions, fine structural details remain elusive. To better understand the structure of RyR1, we have previously used a fluorescence resonance energy transfer (FRET)-based method using a fused green fluorescent protein (GFP) donor and a fluorescent acceptor, Cy3NTA that binds specifically to short poly-histidine 'tags' engineered into RyR1. However, the large size of the GFP fusions and the need to permeabilize cells expressing these constructs (to allow Cy3NTA entry) limits interpretation of the resulting FRET data. To overcome these problems, we used a dodecapeptide sequence containing a tetracysteine (Tc) motif to target the bi-arsenical fluorophores, FlAsH and ReAsH to RyR1. These compounds freely cross intact cell membranes where they then bind covalently to the tetracysteine motif. First, we used this system to conduct FRET measurements in intact cells by fusing a YFP FRET donor to the N-terminus of RyR1 and then targeting the FRET acceptor, ReAsH to an adjacent Tc tag. High levels of energy transfer (~50%) were observed whereas incubation of ReAsH with a YFP-RyR1 fusion protein lacking the Tc tag resulted in no detectable FRET. We also developed a FRET-based system that did not require GFP fusions into RyR1 by labeling N-terminal Tc-tagged RyR1 with FlAsH, a FRET donor and then targeting the FRET acceptor Cy3NTA to an adjacent His tag. A high degree of energy transfer (~70%) indicated proper binding of both compounds to these unique recognition sequences in RyR1. Thus, these two systems provide unprecedented flexibility in FRET-based structural determinations of RyR1. Supported by NIH grant R01AR059124.

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Structural Mapping of Divergent Region Domains in the Type 1 Ryanodine Receptor using Two Complementary FRET-based Approaches

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We used fluorescence resonance energy transfer (FRET) to localize three divergent region domains within the type 1 ryanodine receptor (RyR1), an intracellular Ca^{2+} channel that mediates skeletal muscle excitation-contraction (EC) coupling. Initial cloning studies of the three RyR isoforms identified three ''divergent regions'' of primary sequence dissimilarity spanning amino acids 4254-4631 (DR1), 1342-1403 (DR2) and 1872-1923 (DR3) in RyR1. These regions have been implicated in EC coupling as well as in differential sensitivity to pharmacological agonists. Here, we used permeabilized HEK-293T cells expressing recombinant RyR1 to localize these DRs to the cryo electron microscopic (EM) map of RyR1. First, we measured FRET from a green fluorescent protein (GFP) donor fused to either position 1 or 620 of RyR1, to a FRET acceptor, Cy3NTA, targeted to poly-histidine ''tags'' inserted into DR1 (at position 4429), DR2 (at position1358) or DR3 (at position 1915). While FRET was not detected for His-tagged constructs containing GFP fused at position 1, FRET was observed from GFP fused at position 620 to all 3 His-tagged positions. Second, we targeted a donor to the RyR1 cytoplasmic domain using FKBP12.6 labeled with Alexa Fluor 488, and then measured FRET to Cy3NTA targeted to the His tag sites described above. Donor-FKBPs bound with high-affinity to both recombinant wild type and His-tagged RyRs. FRET was detected from donor conjugated to each of four, well-separated positions on FKBP to Cy3NTA targeted to each divergent region. Since the fused GFPs and FKBP12.6 have already been localized within the cryo EM map of RyR1, we can now triangulate the DR positions to the cryo EM map from these two complementary data sets. Supported by NIH grant R01AR059124 (to JDF, MM, and TG) and R01HL092097 (to RLC).

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The General Anaesthetic Binding Site of Calmodulin Disrupts Ryanodine Peptide Binding

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The skeletal muscle Ryanodine Receptor (RyR1) is a large calcium release channel involved in excitation-contraction coupling. It is also the target for hundreds of disease mutations that cause malignant hyperthermia (MH) or skeletal muscle disorders like central core disease (CCD). MH is typically triggered by volatile anesthetics, but their binding site on RyR1 has not been fully described. RyR1 is under the control of several auxiliary proteins. One of these is Calmodulin (CaM), a Ca^{2+}-binding protein that can suppress RyR1 activity at elevated Ca^{2+} concentrations. Here we investigate how CaM can bind to RyR1, and how this may be affected by volatile anesthetics. We found that CaM can bind to at least three different RyR1 peptides, with the affinity and lobe specificity being altered substantially by the Ca^{2+} concentrations. In addition, we identified two binding sites for sevoflurane, a volatile anesthetic, on Ca^{2+}/CaM. The anesthetic binds to a pocket that is involved in binding RyR1 peptides. In addition, it can alter the affinity of the N-terminal CaM lobe for Ca^{2+}. These findings suggest that binding of anesthetics to CaM may be involved in the pathophysiology of malignant hyperthermia.

2282-Pos Board B301

The Cytoplasmic Foot of RyR1 without the Membrane Spanning Domain Targets Junctionally and Retrogradely Enhances DHPR L-Type Ca^{2+} Currents

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In skeletal muscle, RyR1 (5,037 residues) forms a homo-tetrameric Ca^{2+}-release channel in the sarcoplasmic reticulum (SR), mediates excitation-contraction coupling in response to an orthograde signal from the DHPR in the plasma membrane, and retrogradely enhances L-type Ca^{2+} current via the DHPR. The RyR1 C-terminus forms the Ca^{2+} channel pore across the SR membrane and is believed to be important for inter-subunit interactions, whereas the bulk (~85%) of the protein (the so-called foot) bridges the junctional, myoplasmic gap between the SR and plasma membranes. Here, we have examined the ability of the foot domain (residues 1-4300; $RyR1_{1:4300}$) to target junctionally and interact with the DHPR by expression of a cDNA encoding YFP-$RyR1_{1:4300}$. In dysgenic (α_{1S}-null) myotubes which lack DHPRs, YFP-$RyR1_{1:4300}$ was diffusely distributed and, on the basis of photobleaching, freely mobile within the cytoplasm, consistent with the loss of membrane anchoring. However, after expression in dyspedic (RyR1 null) myotubes (which have DHPRs), much of YFP-$RyR1_{1:4300}$ was immobile within fluorescent foci near the myotube surface, suggestive of junctional targeting and binding to DHPRs. Junctional targeting was confirmed by partial co-localization of YFP-$RyR1_{1:4300}$ and CFP-labeled α_{1S} after co-expression in dyspedic myotubes. Strikingly, YFP-$RyR1_{1:4300}$ was able to retrogradely enhance peak Ca^{2+} current in dyspedic myotubes from 1.6 pA/pF (control) to 6.7 pA/pF, similar to that after expression of full-length RyR1 (7.4 pA/pF). Thus, the isolated, cytoplasmic foot of RyR1 retains the ability to target junctionally and to interact functionally with the DHPR.

2283-Pos Board B302

The Cytoplasmic Foot of RyR1 forms a Stable Homotetrameric Structure Despite Lacking the Membrane-Spanning C-Terminal Domains

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In skeletal muscle, the dihydropyridine receptor (DHPR) in the plasma membrane engages in bi-directional interactions with the type 1 ryanodine receptor (RyR1) in the sarcoplasmic reticulum (SR) such that an ''orthograde'' signal from the DHPR triggers SR Ca^{2+} release via RyR1, and a retrograde signal